The L4F3 Antigen Is Expressed by Unipotent and Multipotent Colony-Forming Cells but Not by Their Precursors

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Antibody L4F3 is a murine monoclonal antibody that recognizes an antigen expressed on in vitro colony-forming cells, including virtually all CFU-GM, CFU-Meg, BFU-E, and CFU-Mix. In the present study we examined whether cells that do not express the L4F3 antigen include precursors of hematopoietic colony-forming cells. Colony-forming cells were depleted from marrow by treatment with L4F3 and complement. The remaining cells generated CFU-GM, BFU-E, and CFU-Mix when cultured in the presence of irradiated adherent cell layers from long-term marrow cultures. Marrow cells not expressing the L4F3 antigen, which were separated by cell-sorting techniques, were depleted of colony-forming cells but nevertheless generated CFU-GM when cultured over irradiated adherent cell layers. These data suggest that there are marrow precursors that do not express the L4F3 antigen and that give rise to colony-forming cells of multiple types. Negative selection techniques should allow the enrichment of these precursors of colony-forming cells, thereby enabling direct studies of these immature stem cells.

SURFACE ANTIGENS expressed by cells of the different hematopoietic lineages change as these cells mature.[1,2] Certain of these differentiation antigens are selectively expressed by cells with different lineage commitments and with greater or lesser proliferative potentials. The maturation-linked expression of antigens by granulocyte/monocyte,[3-18] erythroid,[3,19,20] and megakaryocyte/platelet[3,21-23] elements and their committed precursors[3,24-27] have been identified by numerous monoclonal antibodies. Less is known, however, about antigens expressed by the putatively less mature stem cells that are precursors of in vitro colony-forming progenitors since only indirect assays are possible.

The present study examines the expression of the L4F3 antigen by hematopoietic progenitor cells in vitro including the precursors of colony-forming cells. We have previously shown that the L4F3 antibody reacts with nearly all granulocyte/monocyte colony-forming cells (CFU-GM) and a portion of erythroid burst-forming cells (BFU-E).[3] In this study, using complement (C')-mediated cytolysis and separation of cells stained in indirect immunofluorescence assays by fluorescence-activated cell sorting, we show that the L4F3 antibody also reacts with virtually all megakaryocyte (CFU-Meg) and multipotential (CFU-Mix) colony-forming cells. In contrast, we found that the L4F3 antibody does not react with cells that are precursors for CFU-GM, BFU-E, and CFU-Mix detectable in long-term marrow cultures.

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MATERIALS AND METHODS

Cells

Under an Institutional Review Board–approved protocol, samples of bone marrow obtained from normal healthy adult donors at the Fred Hutchinson Cancer Research Center were collected in heparinized tissue culture medium. Specimens were diluted with an equal volume of RPMI 1640, and bone marrow nucleated cells were separated either as buffy coat cells or by Ficoll-Hypaque (sp gr, 1.077) density gradient centrifugation as previously described.[3]

Monoclonal Antibodies

The generation and characterization of the murine monoclonal antibody L4F3 (IgM) has been previously described in detail.[3] The L4F3 antibody used in the cytotoxicity assays was in the form of unfractionated ascites fluid with a cytotoxic titer of antibody $>10^{-3}$. As a control, ascites fluid containing T11D7, a murine monoclonal IgM of irrelevant specificity (antimouse Thy 1.1), was used (hybridoma clone kindly provided by Dr E. Clark, University of Washington, Seattle). For use in immunofluorescence assays the antibodies were purified and conjugated with fluorescein-isothiocyanate (FITC). Monoclonal antibodies L4F3 and T11D7 were partially purified from ascites fluids by precipitation of IgM with 2% (wt/vol) boric acid.[28] To each 1 mL of ascites fluid 20 mL of 2% boric acid was added dropwise with continued stirring on ice. Stirring was continued for 60 minutes, at which time the solution was centrifuged for 20 minutes, 4 °C, 5,000 × g. The precipitate was dissolved in phosphate-buffered saline (PBS), pH 7.2. When assayed by microzonal electrophoresis, a single band was seen. Partially purified antibodies were then conjugated with FITC (Research Organics, Cleveland) as previously described.[29] The fluorescein/protein ratio of L4F3-FITC was 6.9:1, and that of T11D7-FITC was 7.9:1 as determined by the OD280/OD495 technique.[29]

Tissue Culture

Long-term marrow cultures. Marrow cells were cultured in either 25-cm² tissue culture flasks (Falcon Labware, Oxnard, Calif) at 10^7 per flask or in Ambitube (Miles Scientific, Naperville, Ill) culture tubes at 0.45 to 10×10^6 per tube. As previously described,[30] culture medium consisted of McCoy's 5A medium supplemented with essential and nonessential amino acids and vitamins (GIBCO, Grand Island, NY), 12.5% fetal bovine serum (FBS) (Reheis, Kaukakee, Ill), 12.5% horse serum (GIBCO), 10^{-6} mol/L hydrocortisone (Sigma Chemical Co, St Louis), and 1 mmol/L sodium pyruvate (GIBCO). Cultures were incubated at 37 °C and 5% CO_2

in a humidified incubator for five days and then transferred to a 33 °C, 5% CO_2-humidified incubator. At weekly intervals cultures were fed. After pipetting the supernatant several times, half of the supernatant fluid was removed along with nonadherent cells and an equal volume of fresh medium added. Cells in culture supernatants were assayed for CFU-GM, BFU-E, or CFU-Mix.

Marrow adherent cell layers. Marrow buffy coat cells were cultured at 2×10^7 per 10 mL in 25-cm^2 tissue culture flasks or 3 to 5×10^6 per 2 mL in Ambitube culture tubes as was described. At 3 to 4 weeks after initiation of cultures when a confluent adherent cell layer was present, cultures were irradiated with 1,148 ± 209 cGy from a cesium source (dose rate, 459 = 83 cGy/min). This treatment resulted in cultures that produced no colony-forming cells even when fresh marrow irradiated with the same dose was added (data not shown).

Colony assays. Culture systems for BFU-E and CFU-GM,[3,31] CFU-Meg,[32] and CFU-Mix[31] have previously been described. Cells were cultured at 0.1 to 1.0×10^5 mL, and cultures were counted on day 13 to 15 using an inverted microscope. CFU-GM were identified as colonies with 40 or more cells containing granulocytes, monocytes, or both granulocytes and monocytes. Erythroid bursts were defined as clusters of two or more hemoglobinized colonies after 14 days of culture. These clusters represent progeny of an immature progenitor type.[33] CFU-Mix were colonies containing erythroid and nonerythroid cell types in which there was a single center to the colony. Colonies with two apparent centers, one for erythroid and one for nonerythroid cells, were considered separate colonies and not CFU-Mix. At the cell densities used in these cultures, the majority of CFU-Mix can be expected to be clonal based on studies of G6PD clonality reported by Powell et al.[31]

Cytotoxicity Assay

Cytotoxicity assays were performed as previously described.[3] Briefly, bone marrow cells were suspended at a concentration of 10^7 per mL in RPMI supplemented with 20% (vol/vol) FBS (Hyclone, Logan, Utah) (RPMI–20% FBS) containing a $1:10^3$ dilution of antibody-containing ascites fluid. Cells were incubated for 30 minutes at room temperature (RT), at which time an equal volume of a 1:1 dilution of prescreened baby rabbit serum (a source of complement) in RPMI–20% FBS with 10^2 Kunitz U/mL of DNase (GIBCO) was added. The cells were resuspended and incubation continued for 60 minutes at RT. Cells were washed three times with RPMI and then suspended in RPMI–20% FBS. As a control in these studies, cells were also treated with T11D7, an isotype-identical monoclonal antibody of irrelevant specificity.

Immunofluorescence Assays and Cell Separation by Fluorescence-Activated Cell Sorting

Marrow cells were stained using a 1:20 dilution of directly conjugated L4F3-FITC or T11D7-FITC and then secondarily stained with a 1:20 dilution of an FITC-conjugated goat antiserum to mouse IgM and IgG (Tago, Burlingame, Calif) to enhance fluorescence. All antibodyies were centrifuged at 100,000 g at 25 °C for ten minutes in a Beckman airfuge (Beckman Instruments, San Francisco) immediately prior to use to remove antibody complexes.

Cells stained with either L4F3-FITC or irrelevant control T11D7-FITC were analyzed by flow microfluorometry using a FACS-440 (Becton-Dickinson, Oxnard, Calif). L4F3-stained cells with a fluorescence intensity greater that that of 95% of T11D7-stained control cells were selected as positively stained. Positively and negatively staining cells were separated by fluorescence-activated cell sorting (FACS) at a rate of 2 to 4×10^3 cells/s. Cells were collected into tubes containing RPMI–20% FBS. Collected cells were cultured in colony assays as well as in the long-term cultures over irradiated marrow adherent cell layers.

RESULTS

Inhibition of Colony Formation by Treatment With Antibody L4F3 Plus C'

We used C'-dependent cytolysis to examine the reactivity of monoclonal antibody L4F3 with unipotent and multipotent in vitro colony-forming cells. Treatment with L4F3 plus C' lysed 55% to 75% of marrow nucleated cells when compared with treatment with the control antibody T11D7 as determined by trypan blue dye exclusion in four experiments. Treatment with L4F3 inhibited growth of virtually all immature colony-forming cells including CFU-GM (≥92%), BFU-E (≥95%, bursts with two or more colonies), and CFU-Mix (≥99%) when compared with cells treated with T11D7 (Table 1). In three additional experiments we found that L4F3 treatment also inhibited the growth of CFU-Meg (96% inhibition) (mean ± SEM of 15 replicates per data point: L4F3 + C' = 0.4 ± $0.3/10^5$; T11D7 + C' = 9.8 ± $1.9/10^5$). Thus, these results confirm the previously described lysis of CFU-GM and BFU-E by antibody L4F3 and C'[3] and further demonstrate the effects of both CFU-Meg and CFU-Mix.

Separation of Colony-Forming Cells by FACS

The effects on colony-forming cells in cytolytic assays also may be due to the depletion of accessory cells. Therefore, we used FACS to isolate marrow cells expressing the L4F3 antigen. Since the fluorescence intensity of marrow cells stained using L4F3 in indirect immunofluorescent assays is relatively low,the distinction between cells with low levels of antigen expression and unstained cells is difficult. We therefore enhanced the fluorescent signal using L4F3 that was directly conjugated with FITC followed by FITC-conjugated goat antimouse Ig (GAM) antiserum to stain marrow cells. As a control, cells were stained with FITC-conjugated T11D7 followed by the FITC-conjugated GAM Ig antisera. In these experiments, cells were considered positively stained if they had a fluorescence intensity greater than that of 95% of the T11D7-FITC–stained control cells. Cells were separated into L4F3-positive and -negative populations. In a representative experiment shown in Table 2, the L4F3-stained population contained 76% of all sorted marrow cells. The separated positively and negatively reacting cells as well as unseparated cells were cultured for CFU-GM, BFU-E, CFU-Mix, and CFU-Meg.

The L4F3-positive cells contained virtually all of the CFU-Mix, CFU-Meg, and BFU-E, and the majority of CFU-GM were present in the sorted populations. The separation of the majority of BFU-E including multiple-colony clusters (two or more colonies per burst) was also observed in two additional experiments in which 60% and 80% respectively of sorted BFU-E were in the positive populations (data not shown). These results directly demonstrate the reactivity of the L4F3 antibody with in vitro hematopoietic colony-forming cells, including multipotential and unipotential progenitors.

Effects of L4F4 Plus C' on Long-Term Marrow Cultures

Since treatment of marrow cells with L4F3 plus C' lysed virtually all detectable immature colony-forming cells, we

Table 1. Generation of Colony-Forming Cells in Long-Term Culture System After Lysis With L4F3 + C'

Experiment	Week	Colonies per Culture Tube					
		L4F3 + C'			T11D7 + C'		
		CFU-GM	BFU-E*	CFU-Mix	CFU-GM	BFU-E*	CFU-Mix
I	0	217 ± 62 (92)†‡	0 ± 0 (>99)‡	0 ± 0 (>99)‡	3,779 ± 531	1,445 ± 266	118 ± 59
	1	12 ± 8‡	16 ± 8‡	0 ± 0‡	1,388 ± 165	419 ± 59	71 ± 18
	2	4 ± 4‡	0 ± 0‡	0 ± 0‡	1,447 ± 207	319 ± 59	65 ± 12
	3	176 ± 40	16 ± 16	20 ± 4	272‖	6‖	18‖
	4	216 ± 40§	60 ± 28	40 ± 20	434‖	59‖	41‖
	5	148 ± 16§	52 ± 12	28 ± 8	230‖	47‖	41‖
	6	108 ± 8	16 ± 4	8 ± 4	112‖	18‖	6‖
	7	49 ± 12	2 ± 1	13 ± 6	44‖	0‖	4‖
	8	40 ± 13	8 ± 4	8 ± 2	4‖	0‖	0‖
II	0	100 ± 200 (99)‡	300 ± 200 (95)‡	0 ± 0 (99)‡	8,400 ± 1,200	6,300 ± 900	1,100 ± 200
	1	540 ± 140‡	40 ± 60‡	20 ± 20‡	740 ± 80	560 ± 40	380 ± 20
	2	172 ± 16	96 ± 48	52 ± 24	148 ± 24	340 ± 224	44 ± 4
	3	256 ± 16	36 ± 20	40 ± 12	308 ± 32	124 ± 100	36 ± 16
	4	183 ± 6	23 ± 9	24 ± 14	20 ± 5	0 ± 0	0 ± 0
	5	192‖	16‖	16‖	0‖	0‖	0‖
III	0	190 ± 50 (96)‡	0 ± 0 (99)‡	0 ± 0 (99)‡	4,690 ± 480	3,500 ± 400	250 ± 50
	1	167 ± 27‡	504 ± 116	28 ± 5‡	803 ± 111	336‖	108‖
	2	1,142 ± 33	ND	ND	357 ± 147	ND	ND
	3	657 ± 82	20 ± 7	44 ± 33	550 ± 350	16‖	32‖
	4	765 ± 157	ND	ND	876 ± 141	ND	ND
	5	264‖	ND	ND	136‖	ND	

Abbreviation: ND, not determined.

*Only bursts with two or more erythroid colonies were counted; small, single colonies were not included.

†Percent inhibition of colony growth at week 0 compared with control.

‡Number of colonies in cultures from L4F3-treated cells significantly less than from cultures of control-treated cells at $P < .01$.

§Week 4, $.01 < P < .025$; week 5, $.025 < P < .05$.

‖Results of a single culture plate.

Equivalent numbers of marrow cells were treated with either L4F3 or T11D7 plus C' and used to establish long-term cultures in the presence of irradiated (1,148 ± 207 cGy) 3- to 4-week-old marrow adherent cell layers. Based on pretreatment counts, 3×10^6 cells were cultured per tube in experiment I and 10^7 cells per tube in experiments II and III. Data are the means ± SEM of replicate cultures for each colony type. Total colonies per tube = (colonies/volume) × (volume in tube)/(volume removed).

examined the effect of L4F3 plus C' on progenitor cells in long-term marrow cultures. Marrow cells treated with L4F3 plus C' were cultured in the presence of irradiated confluent allogeneic adherent cell layers from 4-week-old long-term marrow cultures to provide a permissive environment for the proliferation of putative precursors of colony-forming cells.[34] No colony-forming cells were detectable in control cultures either of irradiated adherent cells alone or of irradiated cultures to which irradiated allogeneic marrow cells were added (data not shown).

In these experiments, equivalent numbers of marrow cells were treated with either L4F3 or T11D7 and C', and the remaining cells, without adjustment for cell loss, were placed in long-term cultures and then assayed weekly for colony-forming cells. In representative experiments (Table 1), treatment with L4F3 plus C' deleted greater than 92% of CFU-GM, 95% of BFU-E, and 99% of CFU-Mix when assayed immediately after treatment as compared with control antibody-treated cells. The cultures of L4F3 plus C'-treated cells showed significant and sustained increases in the numbers of CFU-GM, BFU-E, and CFU-Mix detectable in culture over time. After 2 to 3 weeks the numbers of these colony-forming cells (CFU-GM, BFU-E, CFU-Mix) detected in cultures of the L4F3-treated cells were similar to or greater than the numbers of colony-forming cells in the control cultures of T11D7-treated cells. Significantly fewer colony-forming

Table 2. Reactivity of Antibody L4F3 With Hematopoietic Progenitors: FACS

	Percentage of Total Population	CFU-GM	BFU-E	CFU-Meg	CFU-Mix
Unseparated	100	200.0 ± 2.1	56.7 ± 1.8	20.9 ± 1.0	5.0 ± 0.1
L4F3-positive	76	156.0 ± 2.7 (79)*	42.5 ± 1.1 (99)†	11.3 ± 0.4 (97)	2.6 ± 0.2 (100)
L4F3-negative	24	134.0 ± 3.6 (21)	0.8 ± 0.2 (1)	1.2 ± 0.4 (3)	0 ± 0

*Percentage of total colony-forming cells of each type present in sorted populations.

†BFU-E present in cultures of sorted cells were predominantly of the large, single-colony type. In cultures of unseparated cells, bursts with two or more colonies as well as single colonies were present.

Separated and unseparated cells were cultured at 0.83×10^5/mL, and results are displayed as the mean ± SEM of colonies per 10^5 cells for five replicate cultures.

cells were detectable in cultures of L4F3-treated cells in the absence of irradiated marrow adherent cells as compared with those with adherent layers (data not shown).

Immediately following L4F3 treatment, BFU-E of two or more colonies were eliminated, and the only detectable erythroid progenitors formed almost exclusively small single colonies. Virtually all of the BFU-E subsequently detected in the long-term cultures of L4F3-treated cells consisted of two or more erythroid colonies, suggesting the origin of these bursts from less mature progenitors than were demonstrable at the initiation of cultures.[33,35] The CFU-Mix colonies detected in the long-term cultures of L4F3-treated cells were large, and the number detectable increased over the first 2 to 4 weeks of culture. These data suggest that precursors of immature BFU-E and CFU-Mix as well as CFU-GM do not express the L4F3 antigen.

Long-Term Cultures of L4F3-Stained Cells Separated by FACS

In cytolytic assays accessory cells in addition to colony-forming cells may be depleted. Therefore, we isolated L4F3-stained and unstained marrow cells using FACS. The resultant cells, placed in long-term cultures, were analyzed for CFU-GM production as an indicator of precursors of colony-forming cells since CFU-GM correlated with the production of other colony-forming cell types in our culture system. In a representative experiment (Table 3), L4F3-negative, L4F3-positive, and unseparated marrow cells were cultured over irradiated adherent cell layers. After 1 week, the number of CFU-GM detected in cultures of L4F3-negative cells was equivalent to or greater than the number in cultures of L4F3-positive cells despite the fact that the culture of L4F3-positive cells contained almost three times as many CFU-GM at initiation. Moreover, after 1 week, the number of CFU-GM in cultures of L4F3-negative cells was similar to that from cultures of unseparated cells. These results were corroborated in two additional experiments (data not shown). These data confirm that the precursors of colony-forming cells detectable after 3 weeks in long-term culture are not present in L4F3-positive marrow cell populations but are present in the L4F3-negative populations.

Table 3. Generation of CFU-GM in Long-Term Cultures From Isolated L4F3-Negative Cells

Weeks	Colonies per Culture Tube		
	L4F3-Negative	L4F3-Positive	Unseparated
Culture initiation	938 ± 56*	2,652 ± 102*	4,660 ± 117
1	1,344 ± 203	1,017 ± 102	1,127 ± 210
2	231 ± 34	429 ± 66	399 ± 57
3	760 ± 60	467 ± 17	809 ± 97
4	196†	8†	202†

*Mean ± SEM of CFU-GM per culture tube. Bone marrow cells were separated by FACS into negatively stained, positively stained, and unseparated populations. The sorting window for positive cells contained 74% of the analyzed L4F3-stained marrow cells, and the negative window contained 25% of the marrow cells. Ambitubes with established marrow adherent cell layers were irradiated, and 7×10^5 of the L4F3-negative, 1.7×10^6 of the L4F3-positive, and 2.3×10^5 of the unseparated marrow cells were added to separate culture tubes. Single or duplicate cultures for CFU-GM were performed weekly from each individual tube. Colonies per tube = (colonies/volume removed) × (volume in culture tube)/(volume removed).

†Results of a single culture plate.

DISCUSSION

The present study extends our previous characterization of the L4F3 antigen expression by unipotent hematopoietic colony-forming cells to an assessment of multipotent colony-forming cells and, in long-term culture, the putative precursors of colony-forming cells. The L4F3 antigen has a relative molecular weight of 67,000 daltons and is also detected by the MY-9 antibody[24] as determined by competitive binding inhibition and comodulation studies (Andrews and Bernstein, unpublished observations, J. Griffin, personal communication). It is found at barely detectable levels on the surface of mature monocytes and granulocytes in immunofluorescence assays but in greater concentrations on immature granulocytic elements in bone marrow.[3] In the present study, we have shown by cytolysis and cell sorting that the L4F3 antigen is present on virtually all colony-forming cell types in bone marrow including CFU-GM, CFU-Meg, CFU-Mix, and immature BFU-E (two or more colonies/burst).

By culturing separated populations of marrow cells over irradiated allogeneic marrow adherent cell layers,[34] we have found that the L4F3 antigen is not expressed on the precursors of colony-forming cells. Thus, although L4F3 plus C' treatment depleted virtually all immature colony-forming cells (CFU-GM, BFU-E, CFU-Mix), when the remaining cells were cultured over irradiated marrow adherent cell layers, increasing numbers of unipotent and multipotent colony-forming cells were detectable for up to 8 weeks. In the absence of an irradiated adherent cell layer, cultured L4F3-treated cells generated fewer colony-forming cells, and the cultures became senescent in 3 to 4 weeks. The data suggest that marrow cells treated with L4F3 produced more colony-forming cells for a longer time than do control cultures. Thus, it is possible that L4F3-expressing cells may inhibit the proliferation of these precursors of colony-forming cells.

The observation that cultures of L4F3-negative cells generated substantially greater numbers of colony-forming cells than were detectable at initiation strongly suggests that the L4F3-negative cells included precursors capable of substantial proliferative and/or differentiative activity in this culture system. Further, the length of time over which cultures contained detectable colony-forming cells, recent data on the cell cycle characteristics of committed colony-forming cells in long-term cultures,[35] and the cultures of L4F3-positive cells enriched for CFU-GM all suggest that it is unlikely that colony-forming cells detected in long-term cultures of L4F3-treated marrow represented the self renewal of a small number of cells committed to granulopoiesis.

Few other antigens present on immature multipotential as well as unipotential colony-forming cells have been described. Antibodies against HLA-DR determinants have been shown by a number of investigators to recognize CFU-GM, BFU-E, and CFU-Mix.[30,36-39] Non-HLA determinants include the antigen identified by antibodies 12.8,[40]

MY-10,[25] and B1.3.C5,[41,42] which react with a 115,000-dalton antigen. The RFB-1 antibody described by Bodger et al[26] identifies an antigen expressed by CFU-E, BFU-E, CFU-GM and CFU-Mix as well as a subset of peripheral blood T lymphocytes. Ferrero et al[43] described a series of monoclonal antibodies reactive with progenitor cells; however, as in the case of RFB-1, the antigens identified by many of these antibodies have yet to be defined.

Less is known, however, about antigens expressed by the precursors of colony-forming cells. We have recently shown that the 12.8 antigen is expressed by the precursors of colony-forming cells detected in long-term cultures.[40] Ferrero et al[43] also have described antibodies reactive with pre–CFU-GM. Certain, though not all, HLA class II determinants are expressed on these cells,[30,38,39,44] and we now demonstrate that the L4F3 antigen is not expressed by these precursors.

Thus by using selection techniques as described in this paper, the precursors of colony-forming cells can now be separated from in vitro colony-forming cells and mature myeloid and lymphoid hematopoietic elements. The present studies suggest strategies for isolating these precursors that will make it possible to further characterize their requirements for proliferation and/or differentiation.

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